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YEAR 2 PROGRESS REPORT

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GROWTH FACTORS AND TENSION-INDUCED SKELETAL MUSCLE GROWTH

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I. Project's Specific Aims and Progress Toward Goals:

Specific Aim 1. Define the mechanical forces and growth factors necessary to form tissue cultured mammalian skeletal myoblasts into functional organs ("organoids") containing parallel arrays of myofibers organized into muscle fascicles. Primary neonatal mouse skeletal myoblasts, as well as the mouse C2C12 adult skeletal myoblast cell line will be used for these studies. Both normal C2C12 myoblasts and those stably transfected with the recombinant human growth hormone (rhGH) gene will be utilized.

As described in the Year 1 Progress Report, this goal was accomplished during the grant's first year. Two papers are in press describing the techniques for engineering skeletal muscle organoids in vitro from mammalian primary and cell line myoblasts. These new techniques utilize inexpensive materials and large sample numbers can be easily generated (Shansky, DelTatto, Chromiak, & Vandenburgh, 1997; Vandenburgh, Shansky, Del Tatto & Chromiak, 1997). While much time and effort has gone into accomplishing the goals of Specific Aim 1, the new model system developed provides a simplified way for studying tension release muscle atrophy in tissue culture. We believe it to be the tissue culture equivalent of the hindlimb unloading animal model and it may become the standard tissue culture model for ground-based and flight studies of muscle wasting in microgravity.

Specific Aim 2. Study the regulation of protein turnover in mammalian skeletal muscle organoids by tension and defined growth factors such as growth hormone, insulin-like growth factors, androgenic steroids, and glucocorticoids.

Tension release studies over a 6 day period in vitro lead to a 35% ($P<.01$) loss in total cellular protein (Vandenburgh et al., 1996a). The secretion rate of the endogenously produced insulin-like growth factor-1 is increased by increased tension in tissue cultured muscle cells (Perrone, Fenwick-Smith, & Vandenburgh, 1995) and decreased tension on rhGH-secreting myofibers in vivo leads to a 77% decrease in rhGH secretion levels (Vandenburgh et al., 1996a). Thus tension can regulate muscle growth factors produced endogenously from innate or foreign genes. The decrease in rhGH secretion probably does not result from decreased rhGH gene transcription since the gene is under the control of the constitutively expressed retroviral LTR promoter, but from muscle fiber atrophy and down regulation of total protein synthesis.

More recently, we have studied the effect of tension release on myosin heavy chain accumulation and synthesis in primary rodent skeletal muscle organoids in vitro. Tension release lead to a rapid and significant decrease in both myosin heavy chain synthesis and content (**Figure 1**). These results thus establish tension release of skeletal muscle organoids as a good in vitro model for studies on the effects of either exogenously added or endogenously produced growth factors on the atrophy process. Continuation of these studies will be a primary focus of year 3 of the project.

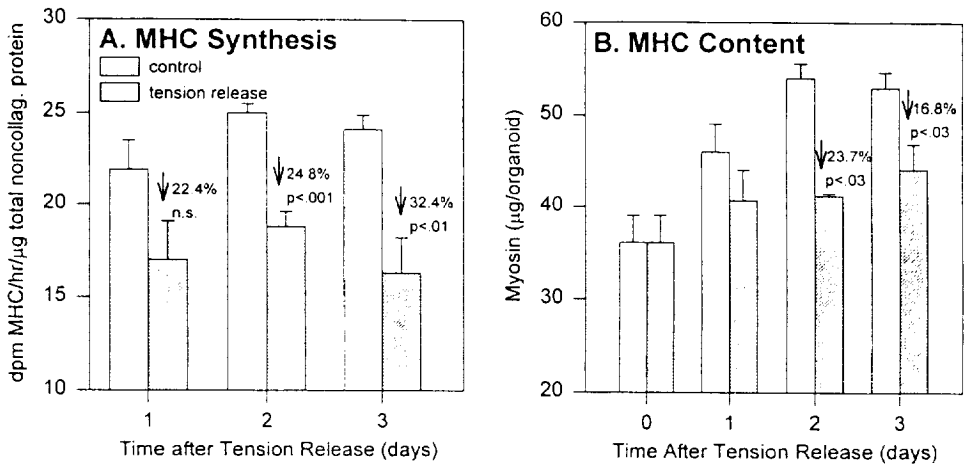

Figure 1. Myosin heavy chain synthesis and accumulation are sensitive to tension in skeletal muscle organoids engineered from primary rat myoblasts. Myoblasts isolated from rat neonates limbs were engineered into skeletal muscle organoids over a 15 day period. Half of the organoids were maintained under tension, while tension was released from the other half by gently detaching them at one end of the culture well; this resulted in a significant decrease in the length of the organoid over the next 24h (data not shown). On days 1, 2, and 3 after tension-release, organoids were harvested following a 4 hr incubation with ^{3}H-phenylalanine. Harvested organoids were sonicated in sucrose buffer and analyzed on 5% SDS-PAGE gels. Myosin heavy chain (MHC) content was determined on Comassie blue stained gels by comparison to known concentrations of MHC standards. MHC synthesis was measured by cutting the MHC bands from the gels, dissolving in gel solubilizer, and liquid scintillation counting radioactivity.

Specific Aim 3. Develop optimal long term (45-60 day) conditions for maintaining the mammalian skeletal muscle organoids in the modified bioreactor cartridges utilized in the Shuttle's Space Tissue Loss Module.

Tissue cultured avian skeletal muscle organoids were successfully used in the Space Shuttle's middeck Space Tissue Loss (STL) Module to study the effects of short-term (9 days) space travel on skeletal muscle atrophy (Vandenburgh et al., 1995; Vandenburgh, Chromiak, Shansky, & Del Tatto, 1996; Vandenburgh, Chromiak, Shansky, & Del Tatto, 1996; Vandenburgh, Chromiak, Shansky, & DelTatto, 1996). A continuous perfusion system such as the STL is critical for the maintenance of the muscle tissue in positive nitrogen balance. Muscle organoids in the STL under non-perfusion conditions atrophied by 75%, most likely due to the accumulation of metabolic waste products in the cellular microenvironment. In preparation for longer duration (30 day) muscle organoid studies in the International Space Station's Cell Culture Unit (CCU), we utilized the procedures described in Specific Aim 1 to tissue engineer mammalian skeletal muscle organoids containing organized differentiated myofibers from either primary rodent neonatal hindlimb muscle or from the murine C2C12 myoblast cell line. Each organoid, with the approximate dimensions of an adult rat soleus, was engineered from 2 to 5 x 10^6 muscle cells and contained approximately 500 µg total non-collagenous protein when fully differentiated. The organoids were transferred to the Cell Growth Chambers of the STL module and continuously perfused at 1.5 ml/min. They were found to be well maintained under these perfusion conditions as indicated by a constant level of glucose utilization (approximately 2.2 mg/organoid/day) for greater than 25 days (**Figure 2A**); the muscle cells

can thus be maintained under perfusion conditions of the CCU. Both serum-containing medium (2.5% v/v horse serum) and a serum free defined medium (MMM) were successfully used in these studies. This will allow the use of defined growth factors to study their ability to attenuate muscle atrophy in long term ISS studies.

Measuring total cellular protein degradation rates by the release of trichloroacetic acid soluble radioactivity from cells prelabelled with ^{14}C phenylalanine prelaunch is linear for 15-20 days in the perfusion cartridges (**Figure 2B**), but not for longer times. This should not present a problem for analyzing the effects of microgravity on the skeletal muscle cells since microgravity does not appear to affect muscle cell protein degradation rates (Vandenburgh et al., 1995; Vandenburgh, Chromiak, Shansky, & Del Tatto, 1996; Vandenburgh, Chromiak, Shansky, & DelTatto, 1996). Specific Aim 3 has thus been completed and a manuscript describing the perfusion techniques has been prepared for publication (Chromiak, Shansky, & Vandenburgh, 1997).

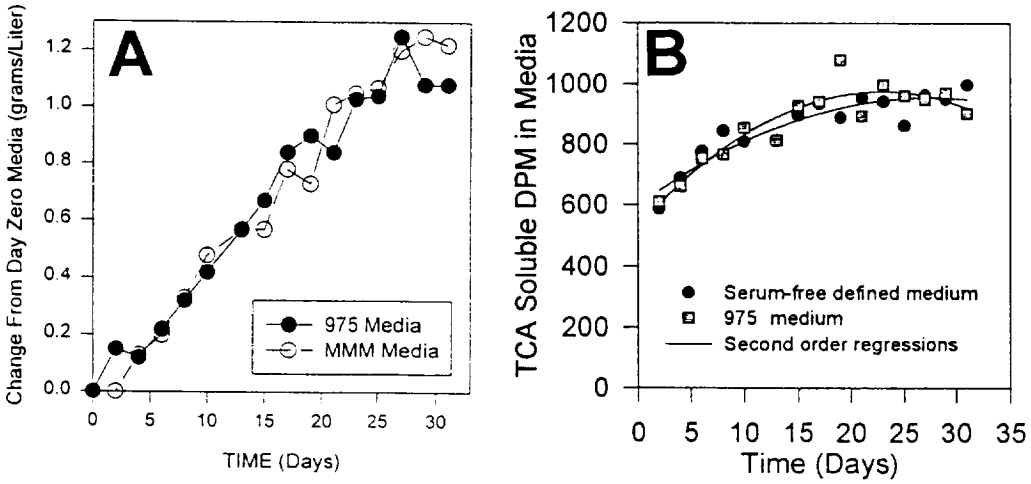

Figure 2. Maintenance of mammalian muscle organoids under ISS-like conditions for 30 days. Myoblasts isolated from rat neonates were formed into skeletal muscle organoids by standard techniques. On Day 19 postplating, the organoids were radioactively labelled with ^{14}C phenylalanine for 36 hr. The organoids were rinsed and transferred under tension to 2 modified bioreactor cartridges of CellCo's QUADMAX™ Perfusion System, the ground-based equivalent of the Shuttle's Middeck Space Tissue Loss (STL) Module. One cartridge was continuously perfused with serum free muscle maintenance medium (MMM) while the other with DMEM containing 2.5% Horse Serum (975/25 medium). Aliquots of medium were removed from the side arm of each cartridge every other day and assayed for glucose/lactate levels to measure cell metabolism (A), and TCA soluble dpms to measure protein degradation rates (B).

Specific Aim 4. Utilize tissue cultured skeletal muscle organoids containing normal mammalian myofibers and C2C12 myofibers stably transfected with the LacZ reporter gene and rhGH gene to determine the long term expression of these genes and rhGH secretion levels in a static and mechanically active tissue culture environment. Determine whether tension-release atrophy in these myofibers is reversed or attenuated by rhGH secretion alone, or in combination with other growth factors and/or mechanical stimulation.

Tissue engineered skeletal muscle organoids from rhGH secreting C2C12 cells have been utilized to determine the effect of tension release on in vivo secretion of rhGH (Vandenburgh et al., 1996a). The ability to modify rhGH secretion from the organoids in

vitro by mechanical stimulation in our mechanical cell stimulator devices (Vandenburgh, 1988; Vandenburgh, 1997), and its effect on muscle atrophy, is currently under investigation in the lab. This will be a major focus of Year 3 along with Specific Aim 2.

Specific Aim 5. Determine the survival, vascularization and innervation of mammalian skeletal muscle organoids following *in vivo* transplantation into syngeneic animals. Determine the *in vivo* expression of transfected genes and the secretion levels of rhGH from C2C12-containing muscle organoids. Determine whether rhGH secretion and/or exercise can attenuate atrophy in hindlimb-suspended host skeletal muscles *in vivo* ("myofiber therapy").

Murine C2C12 skeletal myoblasts stably transduced with the gene for recombinant human growth hormone (rhGH) were tissue engineered in vitro into implantable organoids secreting pharmacological levels of rhGH in vitro. The organoids were subsequently treated with cytosine arabinoside to remove unfused proliferating myoblasts. When implanted subcutaneously under tension into syngeneic C3HeB/FeJ mice, rapid and stable appearance of physiological levels of rhGH in the sera occurred for greater than 12 weeks (Vandenburgh et al., 1996a). The implanted organoids were well vascularized by the host, and retained their gross preimplantation structure, thus allowing easy surgical removal if desired. Removal of the implants led to the rapid disappearance of rhGH from the sera (Vandenburgh et al., 1996a). Delivery of bioactive proteins such as GH from a tissue engineered muscle organ containing only postmitotic cells has several advantages over other forms of muscle based delivery of therapeutic molecules (Blau & Springer, 1995). These include preimplantation monitoring of protein secretion rates, rapid reversibility of treatment, localized delivery to nonmuscle sites, and reduced potential for proliferating cell transformation and tumor formation.

The rhGH released from the muscle implants is biologically active, based on the down regulation of a GH sensitive 20kD protein made in the liver, and secreted as a major urinary protein called MUP (Vandenburgh et al., 1997). MUP is down regulated by injected or infused rhGH in male animals (Norstedt & Palmiter, 1984), and animals implanted with rhGH secreting muscle organoids (**Figure 3A**). Quantitation of the % down regulation of MUPS by cell-based delivery of rhGH showed a 2 fold down regulation of the protein (**Figure 3B**), which lasted as long as the implant remained in the animal. Removal of the implant led to a return of MUP to preimplantation levels (data not shown). Muscle organoids are thus effective "devices" for chronic delivery of proteins such as rhGH and have the potential to deliver proteins such as GH, BMPs, IGF-1 and/or parathyroid hormone (PTH), all of which may be effective in attenuating muscle and bone wasting in microgravity. Recently, we have engineered muscle organoids secreting bone morphogenic protein-6 (BMP-6) to show the ability to deliver other bioactive molecules with this technology (Payumo, 1997).

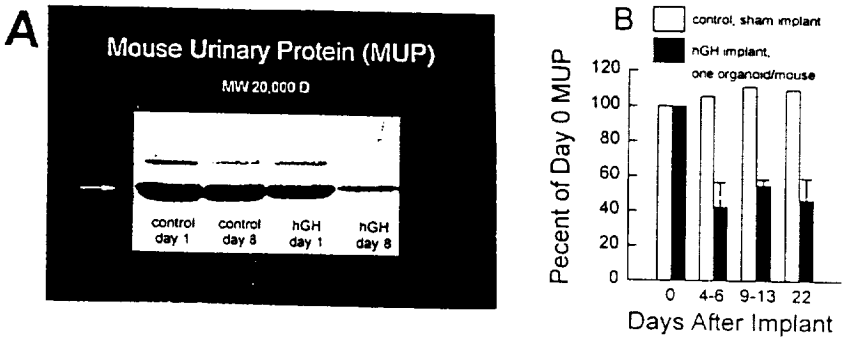

Figure 3. rhGH secreted from muscle organoids is biologically active. (A) Polyacrylamide gel electrophoresis (left) of equal amounts of urine from C3HeB/FeJ mice implanted at Day 0 with either non-rhGH secreting (control) or rhGH-secreting-organoids (hGH); the arrow indicates the position of the 20kD GH-sensitive liver protein MUP (major urinary protein). (B) Comparison of MUP levels in control and rhGH-secreting-organoids (hGH) at three weeks after implantation (Unpublished observations).

We have recently tested whether acute muscle wasting in a hindlimb unloaded mouse model could be reduced by rhGH-secreting muscle organoid implants (Vandenburgh et al., 1997; Vandenburgh, Del Tatto, Shansky, Goldstein, & Yamada, 1997). Initial studies were performed with the plantaris muscle since it is more growth hormone sensitive than the soleus (Grindeland et al., 1994; Aroniadou-Anderjaska, Lemon, & Gilloteaux, 1996). Based on both muscle wet weight (**Figure 4A**) and myofiber cross sectional diameters (**Figure 4B**), animals implanted with rhGH secreting organoids showed significant attenuation of muscle wasting over a 6 day period compared to animals implanted with control non-rhGH-secreting organoids. Similar results have also been obtained in two additional experiments with the less GH sensitive soleus muscle (Figure 4C). While these initial studies need to be extended to biochemical analyses of muscle wasting, they are quite encouraging since injected rhGH has been found to be ineffective by itself in attenuating muscle wasting (Roy et al., 1996; Linderman, Gosselink, Booth, Mukku, & Grindeland, 1994). It's not surprising that continuous delivery of newly synthesized rhGH from a cell based delivery system is more effective than daily rhGH injections since GH has a half life of approximately 15 min in the circulation (Strobl & Thomas, 1994). Cell based delivery of other proteins from skeletal muscle organoids may also be more effective than injected proteins in reversing the wasting effects of microgravity on bone, and will be examined in future ground-based studies. We have thus successfully completed the goals of Specific Aim 5.

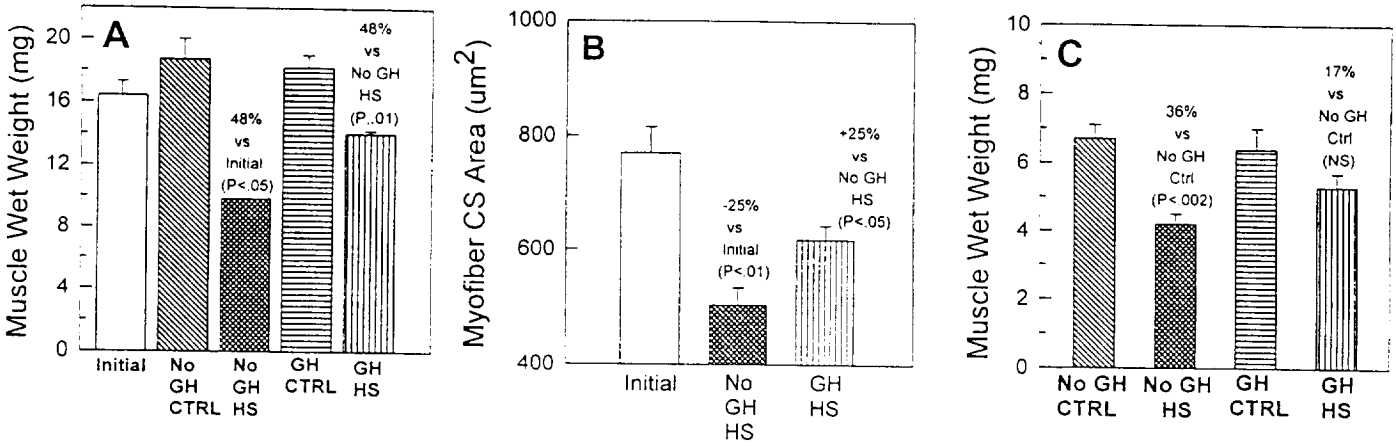

Figure 4. Attenuation of hindlimb unloading-induced skeletal muscle atrophy with rhGH secreting organoids. Six to eight week old C3HeB/FeJ mice were implanted with 2-3 muscle organoids per animal engineered from either normal C2C12 myoblasts or growth hormone (GH)-secreting C2C12 myoblasts. Each rhGH-secreting organoid produced 3 to 5 µg rhGH per day preimplantation and produced a steady state serum level of 2-3 ng/ml from Day 1 to Day 8 after implantation. On Day 1 to 3 after implantation, half of the animals were hindlimb suspended (HS) for 5-8 days (n=3 to 6 per group). Hindlimb muscles were processed for wet weight and myofiber cross-sectioned by standard protocols. (A) and (B) are data for the plantaris muscle while (C) is data for the soleus muscle.

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III. Year 2 Publications

A. Abstracts:

1. Vandenburgh, H.H., Del Tatto, M., Shansky, J., LeMaire, J., Chang, A., Payumo, F., Lee, P., Goodyear, A., & Raven, L. (1996b). Tissue engineered skeletal muscle organoids for reversible gene therapy. In Vitro Cell Dev Biol, 32, 53A.

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3. Vandenburgh, H.H., Chromiak, J., Shansky, J., & Del Tatto, M. (1996). Initial International Space Station (ISS) definition studies for examining the effects of long term space travel on tissue cultured mammalian skeletal myofibers. ASGSB Bulletin 10, 29 (abstract)

4.Vandenburgh, H.H., Chromiak, J., Yamada, S., DelTatto, M., Shansky, J., & Goldstein, L. (1997). Tissue engineered skeletal muscle organoids secreting rhGH attenuate skeletal muscle disuse atrophy. Keystone Symposia on Mol and Cellular Biol, In Press (abstract).

5. Payumo, F.C., Kim, H., Sherling, M., Smith, L., Keeping, H.S., Valentini, R., Drozdoff, V., & Vandenburgh, H.H. (1997). Expression of recombinant human bone morphogenetic protein-6 in C2C12 rnyoblasts. Int Soc Artificial Organs, Submitted

(abstract).

B. Papers

1. Vandenburgh, H.H., Del Tatto, M., Shansky, J., LeMaire, J., Chang, A., Payumo, F., Lee, P., Goodyear, A., & Raven, L. (1996a). Tissue engineered skeletal muscle organoids for reversible gene therapy. Human Gene Therapy, 7, 2195-2200.

2. Shansky, J., DelTatto, M., Chromiak, J., & Vandenburgh, H.H. (1997). A simplified method for tissue engineering skeletal muscle organoids in vitro. In Vitro Cell & Develop Biol, In Press.

3. Vandenburgh, H.H. (1997). Tissue engineering skeletal and cardiac muscle organs with mechanical forces. Engineering in Med and Biol, In Press.

4. Vandenburgh, H.H., Shansky, J., Del Tatto, M. & Chromiak, J. (1997). Methods for the organogenesis of skeletal muscle in tissue culture. In J. Morgan & M. Yarmush (Eds.). Methods in Molecular Medicine: Tissue Engineering (Tottowa, NJ: Humana Press) In Press